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PATENT APPLICATION

for

REDUCING BRANCHING AND ENHANCING FRAGMENTATION IN CULTURING FILAMENTOUS MICROORGANISMS

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REDUCING BRANCHING AND ENHANCING FRAGMENTATION IN CULTURING FILAMENTOUS MICROORGANISMS

Cross-reference to related applications: This application is a continuation of International Application No. PCT/NL99/00395, filed on 25 June 1999, designating the United States of America (International Publication No. WO 00/00613), the entire contents of which are incorporated by this reference.

Technical Field: The invention relates to industrial microbiology, in particular to fermentation technology and especially to fermentation methods for filamentous microorganisms, in particular filamentous bacteria such as actinomycetes. The invention was made in a research program into mechanisms of growth of streptomycetes.

Background: Streptomycetes are Gram-positive, aerobic, filamentous soil bacteria, which belong to the order of *actinomycetales*. In an early stage of *Streptomyces* growth on a solid medium, spores germinate, and subsequently develop into a vegetative mycelium of multi-nucleoidal and branching hyphae with occasional septums (Chater and Losick, 1996). After environmental signals such as nutrient depletion, aseptate aerial hyphae are formed, growing on the vegetative hyphae, the latter being used as a substrate. Eventually, the aerial hyphae form uninucleoidal cells that develop into hydrophobic spores, which are budded off from the tips of the hyphae. One of the striking features of streptomycetes and other members of the order actinomycetales is their ability to produce a wide variety of secondary metabolites, including many antibiotics, which are produced in temporal relation to the onset of morphological differentiation in surface-grown cultures (Chater, 1989; Miyadoh, 1993). The molecular processes regulating the events that lead to differentiation of *Streptomyces* are presently only superficially understood, although new and interesting insights into the genetics of streptomycetes have come to light (reviewed in Champness and Chater, 1993; Chater, 1993).

Most streptomycetes only sporulate on solid media, while growth in liquid cultures is restricted to the formation of vegetative mycelium. This typically develops into intricate networks of hyphae, among others resulting in

pellet formation, with only the most outwardly oriented sections showing high physiological activity, resulting in low yield of the desired product per unit of biomass. Furthermore, because of their filamentous morphology, high density fermentations of biotechnologically interesting streptomycetes often are highly viscous, resulting in a low biomass accumulation due to for instance aeration and mixing problems. From this perspective it is desirable that fragmentation of the mycelium in submerged cultures is stimulated, that branching of the mycelium is reduced and that in general the viscosity of the culture is reduced.

Cell division in all bacteria analysed so far involves the tubulin-like GTP-binding protein FtsZ, which polymerises into a ring at the prospected site of the septum, presumably forming the physical scaffold for the assembly of the cell division apparatus (reviewed in Lutkenhaus and Addinall, 1997). In *Escherichia coli* and *Bacillus* species many factors have been identified that are involved in cell division, but little is known about this process in actinomycetes. Here septum formation does not lead to actual cell division, and while in most bacteria ftsZ is essential, the gene has been shown to be dispensable for mycelial growth in *Streptomyces coelicolor* (McCormick et al., 1994).

In contrast to most actinomycetes, *Streptomyces griseus* shows the ability to sporulate in submerged cultures over a short time period, when grown in defined minimal media (Kendrick and Ensign, 1983; Ensign, 1988). Kawamoto and Ensign (1995a,b) identified a mutation in the gene *ssgA* that relieved repression of sporulation in rich media. *SsgA* encodes an acidic protein with a molecular mass of approximately 5 kDa that displays no significant homology to any other known protein in the database; in the sequenced genome of the actinomycetes *Mycobacterium tuberculosis* and *Mycobacterium leprea* no *ssgA* has been found

(http//kiev.physchem.kth.se/mycdb). Overexpression of *ssgA* resulted in fragmented growth and suppression of sporulation in submerged cultures of *S. griseus*. Fragmented growth was also observed by Kawamoto and Ensign (1995b) by overexpression of *ssgA* in *S.lividans*, which was supposed to have an ssgA of its own on the basis of weak signals on a Southern blot. In *S.griseus*, Western blot analysis with polyclonal antibodies raised against SsgA revealed that expression of SsgA directly correlates to the onset of submerged sporulation, with the protein appearing shortly before spore formation (Kawamoto et al., 1997). Importantly, although sporulation and production of the antibiotic streptomycin are apparently linked in *S. griseus*, no suppression of streptomycin production was observed. Apparently, regulation of sporulation and antibiotic biosynthesis occur via separate pathways.

The present inventors have shown that the activity of SsgA from *S. griseus* is not limited to the organism in which it is found. The activity can advantageously be transferred to other organisms, thereby allowing more fragmented growth and/or reduced branching and/or reduced viscosity of the culture of many filamentous microorganisms, in particular actinomycetes and steptomycetes. This special growth behaviour is observed in a wide variety of culture mediums. It is particularly surprising, that organisms in which a significant endogenous *ssgA*-like activity is not detectable still respond to the presence of the product of the *ssgA* gene. Thus we demonstrate that introduction of *ssgA* into various bacteria, in particular actinomycetes that lack significant endogenous *ssgA* activity results in suppressed branching and enhanced fragmentation of the mycelium in liquid culture, resulting in significantly lower viscosity of culture broths. In addition to autonomously replicating plasmids containing constitutively expressed *ssgA*, we devised

a system that allows easy integration of the gene in the chromosome, with the advantage of high stability combined to that of independent regulation of *ssgA*.

Thus the invention now provides a method for producing a filamentous bacterium showing reduced branching during growth, particularly growth in a liquid medium, comprising providing such a bacterium with the capability of having or expressing heterologous SsgA activity, which activity in *Streptomyces Griseus* is encoded by an *ssgA* gene having the sequence:

1 ATGCGCGAGTCGGTTCAAGCAGAGGTCATGATGAGCTTCCTCGTCTCCGA

51 GGAGCTCTCGTTCCGTATTCCGGTGGAGCTCCGATACGAGGTCGGCGATC

101 CGTATGCCATCCGGATGACGTTCCACCTTCCCGGCGATGCCCCTGTGACC

151 TGGGCGTTCGGCCGCGAGCTGCTGCTGGACGGGCTCAACAGCCCGAGCGG

201 CGACGGCGATGTGCACATCGGCCCGACCGAGCCCGAGGGCCTCGGAGATG

251 TCCACATCCGGCTCCAGGTCGGCGCGGACCGTGCGCTGTTCCGGGCGGGG

301 ACGGCACCGCTGGTGGCGTTCCTCGACCGGACGGACAAGCTCGTGCCGCT

351 CGGCCAGGAGCACACGCTGGGTGACTTCGACGGCAACCTGGAGGACGCAC

401 TGGGCCGCATCCTCGCCGAGGAGCAGAACGCCGGCTGA

As explained above the presence of additional SsgA activity, in particular heterologous SsgA-activity (meaning activity not in a form as present in the microorganism in nature), irrespective of the presence or absence of endogenous SsgA activity, leads to enhanced fragmentation,

reduced branching and thus reduced viscosity in a wide range of culture mediums. The activity may be provided in any suitable manner, but it is preferred that the activity is provided by transfecting or transforming said filamentous bactrium with additional genetic information encoding said activity. Examples of such methods are presented hereinbelow, but the art of genetic engineering of bacteria is so well advanced that persons skilled in the art will be able to come up with numerous methods and variations thereof to provide an intended filamentous bacterium with a gene encoding SsgA-like activity. SsgA-like activity is functionally defined as the ability to enhance septation, fragmentation and/or reduce branching in (typically) submerged cultures of filamentous microorganisms, in particular bacteria, more specifically actinomycetes. The activity of other *ssgA*-like genes or fragments of *ssgA* genes or derivatives of *ssgA* genes which are within the invention must be functionally the same, but that does not mean that the amount of activity per molecule needs to be the same. SsgA-like activity is thus defined as similar in kind, though not necessarily in amount. Other genes encoding such SsgA activity than the genes disclosed herein can be obtained without departing from the invention by applying routine hybridization and/or amplification techniques. Means and methods for expressing such genes are well known in the art so that there is no need to go into detail here regarding cloning vectors, expression vectors, (inducible) promoters, enhancers, repressors, restriction enzymes, etc. etc. For stability of the presence of the added SsgA-activity to the bacterium, in particular for application in large scale fementations, it is however preferred that the genetic information encoding the additional SsgA activity is integrated into the host cell genome. In this case typically the genetic information will be in the form of DNA. However, neither RNA, heteroduplexes or even PNAs are excluded from the present invention as means to provide the additional genetic information to a microorganism. The invention is

preferably applied in the field of filamentous bacteria, in particular actinomycetes and most specifically to streptomycetes. In these embodiments in particular it is preferred to apply *ssgA* genes derived from actinomycetes, especially from other actinomycetes than the one to be altered in growth characteristics. This of course is automatically the case in a bacterium that does not have SsgA activity to any significant amount itself. Using a gene from a related organism enhances the compatibility of the expression machinery of the host with the gene. Thus it is particularly preferred to provide a *Streptomyces* with an *ssgA* (-like) gene from a different *Streptomyces*. *SsgA* genes are found in *Streptomyces griseus*, *Streptomyces collinus*, *Streptomyces albus*, *Streptomyces goldeniensis and Streptomyces netropsis*. It is preferred to provide *Streptomyces* strains not having significant endogenous SsgA activity with a gene from the earlier mentioned strains.

It is useful to ensure that said additional SsgA activity is inducible or repressible with a signal. In this way the growth characteristics of the bacteria can be modified at will. Of course the final goal of the present invention is to enhance the production of useful products by the microorganisms by modifying the microorganisms according to the invention. Useful products produced by or through microorganisms according to the invention include so called secondary metabolites, typically antibiotics or antitumour agents, but also immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, herbicides, antiparasitic agents, ruminant growth promoters, bioinsecticides, receptor (ant)agonists, hetreolgous proteins or even simple biomass. In the case of Streptomycetes such a useful product is typically an antibiotic. It is thus therefore preferred according to the invention to modify antibiotic producing strains of *Streptomyces*, particularly those not displaying a significant

endogenous SsgA like activity, with genetic information encoding SsgA activity. On the other hand the invention can also be very suitably applied to Streptomycetes or other microorganisms expressing heterologous proteins (or overexpressing homologous/endogenous proteins).

For ease of production it is preferred that the useful product, said antibiotic or said protein, is secreted by said bacterium. The protein to be expressed may very well be a protein involved in the pathway of making a useful product such as an antibiotic, so that this production can be further enhanced on top of the improvement by the reduced fragmentation, etc. In that case it would be very suitable to combine the two genes on one vehicle for introduction into the bacterium. The bacteria resulting from the methods according to the invention are of course also part of the invention. They have additional SsgA activity (or are capable of expressing such activity) and they thereby will typically have different growth characteristics than the unmodified microorganisms when said SsgA activity is present. Thus the invention also provides a filamentous bacterium obtainable by a method according to invention. Preferred microorganisms according to the invention are actinomycetes and typically streptomycetes. As stated above it is an important goal of the present invention to improve fermentative production of useful products such as antibiotics. Thus the invention also provides a method for producing an antibiotic or a useful protein comprising culturing a filamentous bacterium according to the invention and harvesting said antibiotic or protein from said culture. The advantages of the invention are most clear when the method of culturing is submerged culture. The invention will be explained in more detail in the following experimental part.

Experimental procedures

Bacterial strains, culture conditions and plasmids

E. coli K-12 strains JM109 (Messing *et al.*, 1981), and ET12567 (MacNeil, *et al.*, 1992) were used for routine sub-cloning. The strains were grown and transformed by standard procedures (Sambrook *et al.*, 1989); transformants were selected in L broth containing 1% (w/v) glucose, and ampicillin at a final concentration of 200 μg ml^{-1}. L broth with 1% glucose and 30 μg ml^{-1} chloramphenicol was used to grow ET12567.

Streptomyces coelicolor A3(2) M145 and *Streptomyces lividans* 1326 (Hopwood *et al.*, 1985) were used for transformation and propagation of *Streptomyces* plasmids. Protoplast preparation and transformation were performed as described by Hopwood *et al.* (1985). SFM medium (mannitol, 20 g l^{-1}; soya flour, 20 g l^{-1}; agar, 20 g l^{-1}, dissolved in tap water) is a modified version of that reported by Hobbs *et al.* (1989) and was used to make spore suspensions. R2YE (Hopwood *et al.*, 1985) was used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants.

For liquid culturing of *Streptomyces* we used YEME medium (Hopwood *et al.*, 1985), Tryptone soy broth (Difco) containing 10% sucrose (designated TSBS), or standard minimal medium (MM; Hopwood *et al.*) with 1% mannitol as carbon source.

Strains used for screening of *ssgA* were *Streptomyces albus* G (ATCC 3004), *Streptomyces ambofaciens* (ATCC 23877), *Streptomyces antibioticus* (ATCC8663), *Streptomyces clavuligerus* (ATCC 27064), *Streptomyces coelicolor* M145, *Streptomyces collinus* (DSM 40733), *Streptomyces fradiae* (CBS 498.68), *Streptomyces goldeniensis* (ATCC 21386), *Streptomyces griseus* (ATCC 23345), *Streptomyces kasugaensis*

(DSM 40819), *Streptomyces lividans*, *Streptomyces mobaraensis* (ATCC 25365), *Streptomyces netropsis* (formerly *Streptoverticilium netropsis*; ATCC 23940), *Streptomyces ramocissimus* (ATCC 27529), and the actinomycetes *Nocardia lactamdurans* (ATCC 27382), *Planobispora rosea* (ATCC 53773), *Saccharopolyspora erythraea* (NRRL 2338).

Plasmids pUC18 (Yanisch-Perron *et al.*, 1985), pIJ2925 (Janssen and Bibb, 1993), and pSET152 (Bierman *et al.*, 1992) were used for cloning experiments. While pSET152 is a conjugative shuttle plasmid, in the experiments described in this study the plasmid and its derivatives were introduced by standard protoplast transformation.

pIJ486 (Ward *et al.*, 1986) and the *E. coli/Streptomyces* shuttle vector pWHM3 (Vara *et al.*) as high copy-number vectors (approximately 50-100 copies per chromosome) in *S. coelicolor*. An expression vector, designated pWHM3-E, was constructed by cloning the 300 bp EcoRI/BamHI fragment containing the *ermE* promoter (Bibb *et al.*, 1994) into pWHM3. Standard procedures were used to isolate plasmid DNA from *E. coli* (Sambrook *et al.*, 1989), and to isolate plasmid and total DNA from *Streptomyces* (Hopwood *et al.*, 1985).

PCR conditions

Polymerase chain reactions (PCRs) were performed in a minicycler (MJ Research, Watertown, MA), using Pfu polymerase (Stratagene, La Jolla, LA), and the buffer provided by the supplier, in the presence of 5% (v/v) DMSO and 200 mM dNTP. No additional Mg^{++} was added to the reaction mixture. The following PCR program was used: 30 cycles of 45 seconds melting at 94°C, 1 minute annealing at 54°C, and 90 seconds extension at 72°C, followed by an additional 10 minutes at 72°C.

Constructs for expression of *ssgA*

A 750 bp DNA fragment containing the *ssgA* gene (Accession D50051) was amplified from the *Streptomyces griseus* chromosome by PCR, using primers ssg1 and ssg2 (Table 1). The PCR fragment was cloned as an *Eco*RI-*Bam*HI fragment in pIJ2925, and further into pWHM3, pWHM3-E, and pSET152, resulting in pGWS1, pGWS2, pGWS3, and pGWS4, respectively (Table 1). For pGWS1 and pGWS3, see also Figure 1. The *S. coelicolor* strain with pGWS4 integrated in the *attP* site on the chromosome was designated *S. coelicolor* GSA1. For pGWS1, pGWS3, and pGWS4 we also made derivatives in which the upstream region of *S. griseus ssgA* was replaced by that of *S. ramocissmus tuf1* (Vijgenboom *et al.*, 1994), which is known to be very efficiently recognized by ribosomes and hence typically results in higher expression; these were designated pGWS1-SD, pGWS3-SD, and pGWS4-SD, respectively.

Southern hybridization and probes

Genomic DNAs used for Southern analysis were isolated according to the method described by Hopwood *et al.* (1985). For high-resolution hybridization experiments, to investigate the presence of *ssgA* in various actinomycetes, genomic DNA was digested with the appropriate enzymes and separated electrophoretically on a 0.7% agarose gel in TAE buffer, using the Gibco BRL 1 kb ladder as DNA size markers. Agarose gels were pretreated and subsequently blotted on Hybond-N$^+$ nylon membranes (Amersham) using 20x SSC buffer as the transfer buffer, basically according to Sambrook *et al.* (1989). Hybridization and washing conditions were described previously (van Wezel *et al.*, 1991). Stripping of blots was done by 30 minutes incubation in 0.4 N NaOH at 65^{0}C and subsequent incubation in 0.1x SSC/0.25 M Tris (pH 6.5). The total removal of the probe was checked by overnight exposure of an X-ray film.

For recognition of *ssgA* in Southern hybridization experiments the 580 bp insert from pGWS5 was [^{32}P]-labelled by the random-prime method (Feinberg and Vogelstein, 1983).

Northern Analysis

RNA samples (approximately 20 µg) were glyoxylated, run in a 1.2% agarose gel in 20 mM sodium phosphate buffer (pH 6.7), and blotted onto Hybond N$^+$ nylon membranes using 30 mM sodium phosphate (pH 6.7) as the blotting buffer. Hybridization with the *S. netropsis ssgA* gene was carried out in 5xSSC, 0.1% SDS, and 1x Blocking reagent (Boehringer Mannheim), O/N at 65°C. Washing occurred until the background was sufficiently low.

Nuclease S1 mapping

For nuclease S1 protection assays, 50 nmol of ^{32}p-end-labelled probe (≈10^4 Cerenkov counts min^{-1}) was hybridized to 20 µg of RNA in 3M Na-TCA at 45°C overnight after denaturation at 70°C. All subsequent steps were carried out as described previously (Strauch *et al.*, 1991).

Computer analysis

The BLAST search engines BlastN, BlastP, and BlastX (Altschul *et al.*, 1990) were used to perform database searches, and the Wisconsin GCG Package (Devereux *et al.*, 1984) for sequence alignments and protein analysis.

Results

SsgA is a unique protein that does not belong to any known protein family

Extensive searches with *S. griseus* SsgA of both the translated nucleotide database and the protein database using the BLAST search engines BLASTX and BLASTP resulted in one relevant hit, namely a partial sequence of *Streptomyces albus* G DNA (Accession M28303) that apparently encodes part of SsgA. This DNA was identified upstream of a ß-lactamase gene (Dehottay *et al.*, 1987), and apparently encodes 67 residues of a putative protein with 86% aa identity to aa 18-84 of *S. griseus* SsgA. The lack of the C-terminal half of the gene suggests that the cloning of this *ssgA* homologue was probably coincidental and the result of a cloning artifact. The cloning and sequencing of the complete gene is described below.

Cloning of *S. griseus ssgA* by PCR

The sequence of *S. griseus ssgA* was published by Kawamoto and Ensign (1995b), and deposited in the EMBL/GENBANK database (D50051). In a recent update the translational start codon was proposed 30 nt downstream of the originally indicated start codon. This ambiguity does not influence the outcome of our experiments. On the basis of protein electrophoresis (SDS PAGE) experiments using over-expressed SsgA and in view of the optimal spacing between ribosome binding sequence and start codon, we believe that the ATG of the 11th triplet of the originally proposed reading frame represents the correct translational start codon (data not shown). This is also supported by phylogenetic evidence from the *ssgA* homologous mentioned below.

The 750 bp DNA fragment generated by PCR amplification of *S. griseus* chromosomal DNA using

oligonucleotides ssg1 and ssg2 was cloned into pIJ2925, resulting in pGWS1 (Table 1). Restriction site and sequence analysis confirmed that the fragment indeed contained *ssgA*.

Southern hybridization reveals *ssgA* in a limited number of streptomycetes

Genomic DNAs isolated from several actinomycetes (see legend to Fig. 2) was digested with *Bam*HI and *Pst*I, submitted to agarose gel electrophoresis and hybridised with the 580 bp insert from pGWS5 harbouring *S. griseus ssgA*, under conditions of low stringency to identify all genes with at least remote similarity to *ssgA*. One hybridising band was observed in the lanes containing *S. collinus*, *S. albus*, *S. goldeniensis*, and *S. griseus* genomic DNAs, and two bands of equal intensity in the lane containing *S. netropsis* DNA (Fig. 2). Under stringency conditions allowing the detection of genes with at least 65% homology to *S. griseus ssgA*, we failed to detect a band corresponding to *ssgA* in all other *Streptomyces* species, including *S. coelicolor* and *S. lividans*, in contrast to a previous Southern analysis by Kawamoto and Ensign (1995b), who used a probe that included *ssgA* flanking sequences from an unrelated genomic DNA region. The duplicity of the signal corresponding to *ssgA* in *S. netropsis* was due to a *Bam*HI restiction site in the gene, as can be deduced from the DNA sequence. We also could not detect an *ssgA* homologue in any of the other actinomycetes checked, namely *Nocardia lactamdurans*, *Planobispora rosea*, and *Saccharopolyspora erythraea*.

Cloning and sequencing of *ssgA* homologues from other streptomycetes

Genomic DNA fragments harbouring *ssgA* homologues from three streptomycetes, namely *S. albus*, *S. goldeniensis*, and *S. netropsis*, were amplified by PCR, using oligonucleotides ssg3 and ssg4. These fragments were cloned as *Eco*RI/*Bam*HI fragments into pIJ2925, and the DNA sequence was determined. Table 2 shows the similarities of the *ssgA* genes and the deduced amino acid sequences. Interestingly, the *S. netropsis* and *S. griseus ssgA* gene products share more than 86% identical amino acids (90% similar), which is high in comparison to 79% (85%) for *S. goldeniensis* SsgA and, strikingly, a poor 63% (71%) for *S. albus* SsgA.

S. griseus and _S. netropsis_ sporulate in liquid cultures

The morphology of the streptomycetes and actinomycetes discussed in this paper was checked by various microscopic techniques. To this purpose, the strains were grown in complex (TSBS) or minimal (MM) liquid medium for three days, and growth characteristics monitored. From these experiments it appeared that only *S. griseus* and *S. netropsis* produced abundant spores in liquid cultures, while *S. goldeniensis* and *S. collinus* showed unusual thickening of the tips of the hyphae, but failed to sporulate under the chosen conditions. Interestingly, while *S. griseus* sporulated only in MM, as was already reported by Kendrick and Ensign (1983), *S. netropsis* sporulated abundantly in TSBS as well as in MM. We believe that the relation between sporulation and the expression of SsgA is of particular interest.

Transcription analysis

Transcription analysis by nuclease S1 mapping showed an accumulation of *ssgA* transcripts in *S. griseus* and *S. netropsis* after nutritional shift-down and at the onset of sporulation. *S. coelicolor* did not sporulate under these conditions. Northern analysis of RNA isolated from *S. coelicolor* M145 after nutritional shift-down or normal growth was carried out, using the *S. netropsis ssgA* gene as the probe. Expectedly, this did not reveal *ssgA* transcripts in *S. coelicolor*.

Expression of *ssgA* in *S. coelicolor* M145 results in reduced branching of the hyphae and fragmented growth

The insert of pGWS1 was cloned into pWHM3 and pWHM3-E, multicopy shuttle vectors that replicate in *E. coli* and *Streptomyces*. The resulting plasmids pGWS2 and pGWS3 (Table 1) were introduced into *S. coelicolor* M145 and correct recombinants were selected by checking the insert lengths of the plasmids. In a control experiment we used pWHM3-E transformants.

Transformants containing pWHM3-E (without *ssgA*) or pGWS2 showed little or no altered morphology in the complex liquid media TSBS, YEME, nor in minimal medium (MM), as judged by phase-contrast microscopy (Fig. 3A). However, hyphae of transformants containing pGWS3 showed strongly reduced branching in complex and minimal medium cultures, resulting in clearly less dense mycelial lumps (Fig. 3B). The vegetative hyphae not only show limited branching, but many of the branches are less than a micron in length. When pGWS3-SD was used instead of pGWS3, the effect was even stronger, with small fragments appearing after approximately 30 hrs, which increased over time (Fig. 4). While MM cultures of *S. coelicolor* typically result in very large mycelial lumps that sediment rapidly (virtually all mycelium precipitates within

one minute when shaking was stopped), MM cultures containing pGWS3-SD transformants showed significantly reduced sedimentation rates, with the majority of the mycelium failing to sediment within five minutes after shaking of the cultures was stopped.

Constitutive expression of chromosomally-integrated *ssgA* also results in fragmented growth

The insert of pGWS3 and pGWS3-SD was cloned in pSET152, a conjugative *E. coli/Streptomyces* shuttle vector, resulting in pGWS4 and pGWS4-SD, respectively. These plasmids were introduced into *S. coelicolor* M145 by standard protoplast transformation, and transformants selected by overlay of the transformation plates with apramycin. Chromosomal integration was checked by Southern analysis, and presence of the complete gene confirmed by PCR using oligonucleotides ssg1 and ssg2. The pGWS4 and pGWS4-SD integrants were designated GSA1 and GSA2. *S. coelicolor* M145 harbouring pSET152 without *ssgA* was used as control strain.

While recombinants containing pSET152 displayed wild-type phenotype, with large mycelial lumps and very few smaller fragments, GSA1 showed limited branching, while the phenotype of GSA2 is much similar to that of *S. coelicolor* harbouring pGWS3-SD, with strongly limited branching, frequent septation and fragmented growth (Fig. 3C). This shows that *S. griseus ssgA* integrated in the *S. coelicolor* chromosome can be expressed at a level high enough to allow fragmentation of *S. coelicolor* mycelium in complex and minimal liquid cultures.

High level expression of *ssgA* in other actinomycetes

The *ssgA* expression vectors pGWS3-SD and pGWS4 were introduced in *S. lividans*, *S. clavuligerus*, and *Sacch. erythraea*, to test the effect of SsgA on the morphology of strains other than *S. coelicolor*. Expression in *S. lividans*

using pGWS3-SD or pGWS4 led to a phenotype much similar to that of *S. coelicolor* harbouring the same plasmids, as was expected since *S. lividans* and *S. coelicolor* are strongly related streptomycetes. Interestingly, expression of SsgA in both *S. clavuligerus* and *Sacch. erythraea* also resulted in reduced branching and increased fragmentation in liquid cultures (Fig. 4), even though morphology of these strains is different from that of *S. coelicolor*.

Thus, it appears that overproduction of SsgA has a strong effect on mycelium morphology in submerged cultures of actinomycetes, irrespective of the presence or absence of endogenous *ssgA-like* activities, with the vegetative hyphae showing much enhanced septation and restricted branching. Furthermore, the ageing cultures showed an increasing degree of fragmentation, resulting in higher culture densities and lower viscosity of recombinant streptomycetes expressing *ssgA*. Comparison of the phenotypes of the two categories of *Streptomyces* strains, namely those displaying *ssgA* activity and those without a significant level, is currently in progress, and could give us more insight into the role of SsgA in *Streptomyces* physiology.

Figure legends

Figure 1. Some of the *ssgA* constructs. Arrows show direction of *ssgA*. P_{ermE}, *ermE* promoter; P_{T7}, T7 promoter. Solid lines represent *ssgA* DNA, broken lines represent plasmid DNA.

Figure 2. Southern hybridization for the detection of *ssgA* in actinomycetes. All numbered lanes contain *Bam*HI/*Pst*I-digested chromomal DNA. Marker lanes **(M)** contain 1 kb DNA ladder. Blots were hybridized with the 580 bp insert from pGWS5 as probe, and subsequently with a small amount of radioactively labelled 1 kb ladder.

A. Lanes: 1. *S. coelicolor* 2. *S. lividans* 1326 3. *S. lividans* TK24 4. *S. griseofuscus* 5. *S. fradiae* 6. *S. ramocissimus* 7. *S. collinus* 8. *S. kasugaensis* 9. *S. antibioticus* 10. *Sacch. erythraea* 11. *N. lactamdurans* 12. *P. rosea* 13. *S. griseus*

B. Lanes: 1. *S. albus* 2. *S. ambofaciens* 3. *S. coelicolor* 4. *S. clavuligerus* 5. *S. collinus* 6. *Sacch. erythraea* 7. *S. goldeniensis* 8. *S. mobaraensis* 9. *S. netropsis* 10. *P. rosea*

Figure 3. Phase-contrast microscopy of *S. coelicolor* M145 containing (A) pGWS2, and (B) pGWS3 at 200x magnification, (C) *S. coelicolor* M145 with chromosomally integrated pGWS4 (magnification 500x); upper part, details revealed by electron microscopy (magnification 10.000x)

Figure 4. Phase-contrast microscopy of *S. clavuligerus* ATCC 27064.
(A) *S. clavuligerus* (wild type), (B) Recombinant *S. clavuligerus* containing pGWS4-SD.

Figure 5. Sequences of different *ssgA* genes and proteins from different strains and oligonucleotides.

Oligonucleotides

primer		Nucl. Pos.
ssg1	5' GGCGAATTCGAACAGCTACGTGGCGAAGTCGCCA 3' (*Eco*RI)	-194/-170
ssg2	5' GTGGGATCCGTGCTCGCGGCGCTGGTCGTCTC 3' (*Bam*HI)	+539/+517
ssg3	5' GGGAATTCCATATGCGCGAGTCGGTTCAAGCA 3' (*Eco*RI *Nde*I)	-30/-10
ssg4	5' CCGGTCAGCCGGCGTTCTGCTCCTC 3'	+412/388

Plasmids

pIJ2925	Derivative of pUC19, with *Bgl*II sites flanking the slightly altered multiple cloning site.	Janssen and Bibb, 1993
pWHM3	Multi-copy *E. coli/Streptomyces* shuttle vector. Carries thiostrepton resistance marker	Vara *et al.*
pWHM3-E	pWHM3 with the 300 bp fragment containing the constitutive *ermE* promoter for gene expression	this study
pSET152	*E. coli/Streptomyces* shuttle vector that allows integration in the _C31 attachment site on the *Streptomyces* chromosome. Carries apramycin resistance marker.	Bierman *et al.*, 1992
pGWS1	pIJ2925 containing the 750 bp *ssgA* PCR (ssg1/ssg2) product	this study
pGWS1-SD	pGWS1 with the upstream region of *ssgA* replaced by nt -1/-70 of *S. ramocissimus tuf1*	this study
pGWS2	pWHM3 containing the *Eco*RI/*Hin*dIII insert from pGWS1	this study
pGWS3	pWHM3-E containing the *Bgl*II/*Hin*dIII insert from pGWS1	this study
pGWS3-SD	pWHM3-E containing the *Bgl*II/*Hin*dIII insert from pGWS1-SD	this study
pGWS4	pSET152 containing the *Eco*RI/*Pst*I insert from pGWS3	this study

pGWS4-SD pSET152 containing the *Eco*RI/*Pst*I insert from pGWS3-SD this study

pGWS5 pIJ2925 containing the 580 bp *ssgA* PCR (ssg3/ssg2) product cloned *Eco*RI/*Bam*HI.

Table 1. Oligonucleotides and *ssgA* constructs. Nucleotide positions refer to the location of the primers in respect to the first nucleotide (+1) of the ATG translational start codon of *ssgA*. Underlined sequences indicate non-homologous sequences added to create restriction sites (in italics) at the ends of the PCR fragments.

	S. albus	*S. goldeniensis*	*S. griseus*	*S. netropsis*
S. albus	X	75.2	74.5	72.3
S. goldeniensis	71.3 (75.7)	X	77.5	75.7
S. griseus	66.2 (71.3)	78.7 (85.3)	X	83.3
S. netropsis	63.2 (70.6)	77.9 (83.8)	86.0 (90.4)	X

Table 2. DNA and deduced protein sequence homologies of *ssgA* homologues. Above the diagonal: DNA sequence identities (%). Below the diagonal: protein sequence identities (similarities between brackets).

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